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REVIEW

Plakophilin-2 induced EGFR phosphorylation: a focus on the intracellular activators of EGFR

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The oncogenic role of EGFR in many tumors has attracted a great deal of attention in the recent years and initiated the development of several potent EGFR inhibitors, which are used clinically for cancer treatment. However, the current therapeutic inhibition of EGFR signaling is limited to monoclonal antibodies that bind to the EGFR extracellular domain or tyrosine kinase inhibitors that block EGFR kinase activation directly. Despite the great promise of these inhibitors, a certain percentage of patients develop resistance to these therapies, highlighting the necessity for alternative therapeutic strategies based on our most current knowledge of the mechanisms of EGFR signaling. We recently reported that Plakofilin-2 (PKP2) is a novel ligand-independent cytoplasmic activator of EGFR signaling. Here we focus on recent studies demonstrating important roles of intracellular EGFR activators, and propose targeted disruption of these activators as a novel avenue of therapeutic intervention to inhibit EGFR-mediated cancer development.

Keywords: EGFR dimerization; Cytohesins; SFKs; PKP2

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Introduction

The epidermal growth factor receptor (EGFR) regulates cell proliferation, differentiation, survival, motility, and blood vessel formation [1, 2]. The signaling pathways of epidermal growth factor (EGF) via EGFR are well studied due to their importance in regulating a multitude of biological functions [3-7].

Aberrant EGFR activation correlates with poor clinical outcome and is an important contributor to oncogenic processes. In addition, perturbations to the genetic sequence of EGFR often results in deregulation of EGFR signaling and is common in cancer cells and correlates with neoplastic progression [8, 9].

Current anti-EGFR therapies, such as tyrosine kinase inhibitors (TKIs) and monoclonal antibodies, demonstrate anti-tumor activity in various tumor types including non-small-cell lung cancer (NSCLC), squamous cell carcinoma of the head and neck (SCCHN), and colorectal cancer [10-14]. While both TKIs and monoclonal antibodies against EGFR inhibit the EGFR signaling pathway, they elicit their effects via unique mechanisms. Monoclonal antibodies inhibit EGFR activity by binding to the extracellular region of the receptor, therefore disrupting ligand binding and preventing downstream signaling. Conversely, TKIs inhibit the downstream signaling of EGFR by binding to the intracellular tyrosine kinase domain.

EGFR signaling has been heavily studied, and the promise of targeting this pathway promoted the development of

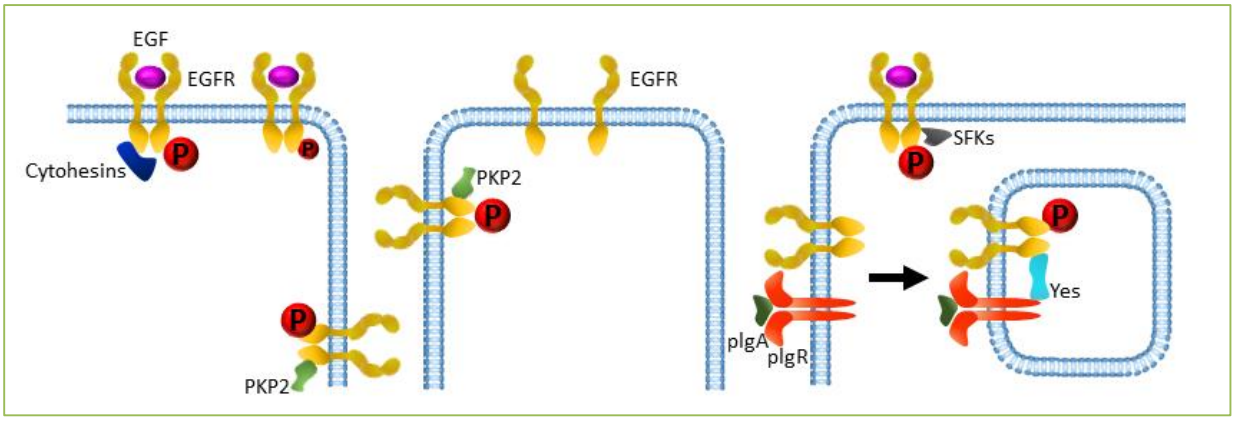

Figure 1. EGFR is activated by intracellular molecules. Cytohesins enhance EGFR activation by directly interacting with the cytoplasmic domains of dimerized receptors and facilitating conformational rearrangements of these domains. PKP2 activates EGFR by facilitating EGFR dimerization even in the absence of ligand stimulation. SFKs directly phosphorylate limited sites on EGFR.Yes-mediated EGFR phosphorylation occurs on the endosome as well, and requires EGF stimulation and pIgA-pIgR transcytosis.

several inhibitors which are now used clinically. However, as with many targeted cancer therapies, many patients eventually develop resistance. Consequently, there is an imminent need to broaden our understanding of the mechanisms of EGFR signal activation in order to develop alternative strategies to target this pathway.

Therefore, in this review, we discuss the novel concept of therapeutic intervention against cytoplasmic EGFR activators as a potential second generation of EGFR related cancer therapies (Figure 1).

Cytohesins are EGF-dependent cytoplasmic EGFR activators

Cytohesins have been shown to be guanine nucleotide exchange factor (GEF) for ADP ribosylation factors (ARF) [15]. The cytohesin family is comprised of four members of high homology: cytohesin-1, cytohesin-2 (ARNO), cytohesin-3 (Grp1), and cytohesin-4 [16, 17]. This family of proteins contain a coil at the N-terminus, followed by a Sec7 domain, and a pleckstrin homology (ph) domain at the C-terminus [16]. Bill *et al* reported that cytohesins, especially cytohesin-2 (ARNO), increase EGFR activation via direct association with the cytoplasmic regions of dimerized receptors and promoting these domains to undergo conformational rearrangements [18]. In addition, the cytohesin-specific antagonist SecinH3 diminishes growth of the EGFR-dependent lung cancer in a xenograft model using PC9 cells. Interestingly, although SecinH3 has been found to target the Sec7 domain of the cytohesins, which is required for GEF catalytic activity, the Sec7 domain of cytohesin-2 activates EGFR independently of its GEF activity [18]. This suggests that the specific binding between the small molecule SecinH3 and the Sec7 domain of cytohesins, rather than its enzymatic activity, is important for SecinH3-driven inhibition of EGFR activation. Importantly, cytohesins do not affect receptor dimerization, but function as activators of dimerized receptors by promoting conformational changes after EGF stimulation. High expression levels of cytohesin-1 and cytohesin-2 (ARNO) overexpression correlate with enhanced EGFR signal activation in human lung adenocarcinomas [18]. Recently, Bill *et al* also reported that inhibition of cytohesins has an anti-proliferative effect against H460 and A546, two gefitinib-resistant lung cancer cell lines [19]. Other studies by Pan T *et al* have also demonstrated that inhibition of cytohesins, with the antagonist SecinH3 or via knock-down of ARNO by ARNO-siRNA, can decrease EGFR activation in the HT29 colorectal cancer cell line [20]. Now, cytohesins are being proposed as novel effective targets for inhibiting invasion and metastasis, and for colorectal cancer patients that developed resistance to Cetuximab or Panitumumab [20].

Src family tyrosine kinases phosphorylate EGFR directly

It is well established that SFKs (a proto-oncogenic cytosolic Src family tyrosine kinases) including c-Src, Lyn and Yes are required for fully activating EGFR signaling [21, 22].

Elevated Src kinase activity is observed in several solid tumors, such as breast cancer [23-25]. Src is known to be involved in the signaling and cross talk between several mitogenic pathways, such as the ER (Estrogen Receptor) and EGFR family signaling pathways [26]. Furthermore, EGFR-mediated Src activation promotes heparin-binding EGF-like growth factor (HB-EGF) shedding from the surface of cells by ADAM family proteases, which drives autocrine EGFR signaling [27]. Since SFKsare involved in many

oncogenic processes including growth and proliferation, invasion, angiogenesis, and metastasis, several Src inhibitors (eg. Dasatinib) are now emerging [28-30]. However, Src expression alone is not fully responsible for transformative ability; herefore, the necessity for combination therapy with other inhibitors such as EGFR inhibitors is recognized [31, 32].

YES is known to be highly expressed in adult neurons, spermatozoa, platelets, and epithelial cells [33, 34]. The expression and kinase activity of Yes and Src are high in malignant skin and colon cancer cells [35, 36]. Su *et al* revealed that binding of the polymeric immunoglobulin A (plgA) to polymeric immunoglobulin receptor (plgR) activates Yes, followed by EGFR signal activation by direct phosphorylation of EGFR *in vivo*, as well as *in vitro* [37]. Su *et al* also discussed that abnormaly produced IgA complexes by mucosal infection might cause excessive activation of EGFR signal activation leading to pathological proliferation, a hallmark of IgA nephlopathy. In this model, Yes-mediated EGFR phosphorylation occurs on the endosome, results in a mild increase in phosphorylation on limited sites (Tyr 845, Tyr 992, and Tyr 1173), and requires EGF-mediated stimulation and pIgA-pIgR transcytosis, suggesting that Yes does not affect receptor dimerization. It has also been reported by other groups that SFKs, including Yes, phosphorylate Y845 and Y1101 of EGFR.Based on current findings, the mechanisms of SFKs-mediated site-specific EGFR phosphorylation still remains unclear and needs to be investigated in the future.

PKP2, a novel desmosomal protein for EGFR dimerization

The plakophilins are members of the armadillo-repeat family. This family is comprised of three different proteins (PKP1, PKP2, and PKP3) [38]. They contain a basic N-terminal head domain, an armadillo repeat (arm-repeat; 42-amino acid repeats) containing region, followed by a small C-terminal tail [38]. Plakophilins contain a conserved sequence in the amino-terminal head domain termed the HR2 domain. PKP2 was initially isolated and believed to be a desmosomal protein, but further studies have demonstrated that it is also localized in the cytoplasm and nucleus [38, 39]. PKP2 mutations are correlated with cardiac disorder arrhythmogenic right ventricular cardiomyopathies (ARVC/D). Additionally, PKP2 knockout mice suffer from embryonic lethality (around E10.5) and display abnormal heart morphogenesis, indicating that PKP2 has critical roles in heart development and function [40, 41].

PKP2 has also been implicated in promoting oncogenesis and it has been reported that PKP2 expression is higher in various cancers, especially at their metastatic stage [42-49]. However, the role of PKP2 in the development of cancer, as well as cancer progression, was unclear. Recently, we showed thatPKP2, but not PKP1 or PKP3, specifically associates with the cytoplasmic region of EGFR [50, 51]. The N-terminus of PKP2, not including the conserved HR2 domain, is critical for this interaction, which results in enhanced EGFR autophosphorylation and EGFR signal transduction. An earlier study indicated that PKP2 localization to desmosomal regions of cell-to-cell attachment depends on the N-terminal portion of PKP2 [39]. This suggests that PKP2 activates EGFR signal pathways in regions of the cell desmosome. More importantly, we demonstrated that PKP2 enhances EGF-independent dimerization and phosphorylation of EGFR, as well as the activation of the downstream effectors of EGFR. PKP2-induced phosphorylation of EGFR and subsequent ERK activation was entirely inhibited by the EGFR inhibitor lapatinib, which suggests that PKP2 exclusively mediates EGFR signal activation in EGFR expressing cancer cells. We further demonstrated that knock-down of PKP2 significantly reduced EGF-induced EGFR autophosphorylation, recruitment of adaptor molecules such as SHP-1 and Grb2 to EGFR, and receptor internalization (unpublished data for internalization). In addition, reduced expression of PKP2 in the breast cancer cell line, MDA-MB-231, impaired their proliferation and metastatic ability, and ultimately reduced the development of tumors *in vivo.*

Since EGFR is known to still be able to dimerize in the absence of EGF stimulation, this PKP2-induced EGF-independent EGFR activation might be one of the mechanisms for this event [52].

As described in this review, SFKs, cytohesinsand PKP2 all directly interact with EGFR and activate downstream signaling. However, their mechanisms in regulating EGFR signaling do not overlap. SFKs are the kinases that phosphorylate limited sites on EGFR. Cytohesins function after ligand-induced EGFR dimerization. In contrast, PKP2 activates EGFR by facilitating EGFR dimerization even in the absence of ligand stimulation.

Future directions in research

Many advances have been made over the past decade in effort to target cancers with aberrant EGFR expression and signaling. The promise of anti-EGFR antibodies and EGFR inhibitors was undermined by patients who did not respond to these treatments due to intrinsic resistance, as well as those who developed resistance over time. Therefore, exploring further into the mechanisms of EGFR activation would provide novel candidate targets, such as cytoplasmic activators of EGFR, which could be inhibited or disrupted.

Additionally, combinatorial therapies targeting extracellular and intracellular mechanisms of EGFR activation could be explored. Although there are many obstacles due to the difficulties in targeting cytoplasmic proteins, emerging small molecules such as SecinH3, or others which can disrupt protein-protein interactions such as stapled α-helical peptide technology [53, 54], could be utilized to target cytoplasmic activators of EGFR described in this review and those yet to be discovered.

Conflicting interests

The authors have declared that no competing interests exist.

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